"Is transplantation of a few leukemic cells inside an artificial ovary able to induce leukemia in an experimental model?"

Soares, Michelle ; Saussoy, Pascale ; Sahrari, Karima ; Andrade Amorim, Christiani ; Donnez, Jacques ; Dolmans, Marie-Madeleine

Abstract

PURPOSE: To evaluate the tumor-inducing ability of a few leukemic cells xenotransplanted inside an artificial ovary. METHODS: Ten and 100 BV-173 leukemic cells were embedded in a fibrin matrix along with 50,000 human ovarian stromal cells, and grafted to the peritoneal bursa of 5 and 5 SCID mice respectively. Four mice grafted with 3x10(6) leukemic cells in fibrin served as positive controls. At 20 weeks post-transplantation, the grafts, liver, spleen, blood and bone marrow were analyzed for the presence of leukemia by anti-CD79α IHC, flow cytometry (FC) and PCR. RESULTS: All mice grafted with 3x10(6) cells developed peritoneal masses 4 weeks after xenotransplantation, and systemic disease was confirmed by IHC, PCR and FC. Among mice grafted with 10 or 100 leukemic cells, none showed any sign of leukemia after 20 weeks, and IHC, FC and PCR on the different recovered tissues all proved negative. CONCLUSION: This study investigates the tumor-inducing potential of a few leukemic cells ...

Document type : *Article de périodique (Journal article)*

J Assist Reprod Genet (2015) 32:597–606
DOI 10.1007/s10815-015-0438-x

FERTILITY PRESERVATION

Is transplantation of a few leukemic cells inside an artificial ovary able to induce leukemia in an experimental model?

Michelle Soares · Pascale Saussoy · Karima Sahrari · Christiani A. Amorim · Jacques Donnez · Marie-Madeleine Dolmans

Received: 15 October 2014 / Accepted: 13 January 2015 / Published online: 4 February 2015

Abstract
Purpose To evaluate the tumor-inducing ability of a few leukemic cells xenotransplanted inside an artificial ovary.
Methods Ten and 100 BV-173 leukemic cells were embedded in a fibrin matrix along with 50,000 human ovarian stromal cells, and grafted to the peritoneal bursa of 5 and 5 SCID mice respectively. Four mice grafted with $3x10^6$ leukemic cells in fibrin served as positive controls. At 20 weeks post-transplantation, the grafts, liver, spleen, blood and bone marrow were analyzed for the presence of leukemia by anti-CD79α IHC, flow cytometry (FC) and PCR.
Results All mice grafted with $3x10^6$ cells developed peritoneal masses 4 weeks after xenotransplantation, and systemic disease was confirmed by IHC, PCR and FC. Among mice grafted with 10 or 100 leukemic cells, none showed any sign of leukemia after 20 weeks, and IHC, FC and PCR on the different recovered tissues all proved negative.
Conclusion This study investigates the tumor-inducing potential of a few leukemic cells grafted inside an artificial ovary. Transplantation of 100 leukemic cells appears to be insufficient to induce leukemia after 20 weeks. These results in an immunodeficient xenografting model are quite reassuring. However, for clinical application, follicle suspensions must be purged of leukemic cells before grafting, as even the slightest risk should be avoided.

Keywords Artificial ovary · Leukemia · Ovarian tissue cryopreservation · Ovarian follicles · Malignant cell purging · Minimal disseminated disease · Follicle isolation

Capsule The tumor-inducing ability of a few BV-173 leukemic cells was evaluated in an artificial ovary by xenotransplantation. One hundred BV-173 cells proved insufficient to induce disease in mice after 20 weeks.

Electronic supplementary material The online version of this article (doi:10.1007/s10815-015-0438-x) contains supplementary material, which is available to authorized users.

M. Soares · K. Sahrari · C. A. Amorim · M.-M. Dolmans
Pôle de Recherche en Gynécologie, Institut de Recherche Expérimentale et Clinique, Université Catholique de Louvain, Brussels, Belgium

P. Saussoy
Département de Biologie Clinique, Université Catholique de Louvain et Cliniques universitaires Saint-Luc, Brussels, Belgium

J. Donnez
Société de Recherche pour l'Infertilité, Brussels, Belgium

M. Soares · M.-M. Dolmans (✉)
Département de Gynécologie, Cliniques Universitaires Saint-Luc , Av Hippocrate 10, 1200 Brussels, Belgium
e-mail: marie-madeleine.dolmans@uclouvain.be

Introduction

Early detection and increasingly effective anti-cancer therapies have led to a rise in post-cancer survival rates in recent decades. Today, the 5-year survival rate after childhood and adolescent cancer stands at around 82 % [1, 2]. In the United States alone, an estimated 379,112 survivors of childhood and adolescent cancer (diagnosed between birth and 19 years) were alive as of January 1, 2010 [2]. For these young patients, recovery of ovarian function and reproductive potential after treatment have become important quality-of-life issues. Various chemotherapeutic agents, particularly alkylating agents, have been identified as gonadotoxic, and pelvic or total body irradiation (TBI) can cause irreversible gonadal damage [3–5], potentially leading to infertility problems and premature ovarian failure (POF) [3–9]. This risk depends on the patient's age and follicle reserve, as well as the type and dose of drug received, and is not always easy to predict. The risk of relapse

and potential need for more aggressive treatments should also be taken into account [10], so that adequate fertility preservation options can be offered to these patients before starting cancer treatment.

Leukemia is the most common malignant disease in childhood (0–14 years), responsible for almost one-third of all tumors diagnosed at this age [11]. Hematological malignancies represent the most frequent indication for ovarian tissue cryopreservation (OTC) in our department, with leukemia accounting for 11 % of procedures [12].

Acute lymphoblastic leukemia (ALL) patients are at low risk (<20 %) of POF, while those with acute myeloblastic leukemia (AML) are at medium risk (20–80 %), according to the classification by Wallace et al. [4] based on current treatments. Some of these patients will, however, switch to the high-risk category (>80 % POF) in case of relapse or incomplete response to treatment. Indeed, changing categories occurred in around 21 % of patients (3/14) with acute leukemia in one report [10].

Leukemia patients are often prepubertal and generally require immediate chemotherapy that cannot be delayed, so embryo and oocyte cryopreservation or fertility preservation options involving ovarian stimulation cannot be proposed to these young women [13–16]. OTC before treatment is the only available option in these cases.

However, the safety of retransplantation of frozen-thawed ovarian tissue in case of hematological malignancy remains of great concern because of the risk of returning malignant cells back to the patient. Several studies have identified malignant cells in cryopreserved ovarian tissue of leukemia patients by sensitive PCR [17–19] and flow cytometry [20] analysis. In one study, the tumor-inducing potential of leukemic cells present in ovarian tissue was demonstrated in a xenografting model [18]. In another study, however, ovarian tissue from patients in complete remission did not appear to contain any viable malignant cells; although PCR results were positive, none of the grafted mice developed the disease [21]. Given the presence of leukemic cells and the uncertainty of disease transmission, reimplantation of ovarian tissue in young women with the acute form of leukemia is not currently recommended [13, 22, 23].

For this reason, our team has been working on the grafting of individual ovarian follicles, enzymatically isolated from frozen-thawed ovarian tissue and embedded in 3-D matrices [24–28]. Addition of ovarian stromal and endothelial cells to the matrix was found to be crucial for the formation of a well vascularized ovary-like structure [29]. This artificial ovary provides a more natural environment for follicle survival and development, and the first studies are encouraging [25, 28].

A technique for isolation of human preantral follicles following good manufacturing practice (GMP) conditions was recently optimized by our team with a view to clinical application [30]. In this setting, however, isolated follicles would be retrieved from cryopreserved ovarian tissue that could possibly be contaminated with cancer cells. A study was therefore conducted to evaluate the safety of our follicle pick-up technique when leukemic cells were added to digested ovarian tissue suspensions (Soares et al., submitted). We detected leukemic cell contamination of our follicle suspensions after pick-up, raising questions about the capacity of a few leukemic cells to reintroduce the disease. The present study was initiated to investigate the leukemia-inducing potential of a few leukemic cells grafted in an artificial ovary environment using an immunodeficient mouse xenografting model. Leukemic cells were embedded in a fibrin matrix along with ovarian cells (essentially stromal and endothelial cells) in order to study leukemic cell survival and proliferation in conditions resembling the artificial ovary environment as closely as possible.

Materials and methods

Ten and 100 leukemic cells were embedded in a fibrin matrix along with ovarian cells and grafted to the peritoneal bursa of 5 and 5 immunodeficient mice respectively for 20 weeks. Four mice grafted with 3 million BV-173 cells inside a fibrin matrix served as positive controls.

Ovarian tissue digestion and stromal cell isolation

Human ovarian biopsies were taken in order to retrieve ovarian cells to embed in the matrix together with leukemic cells. Only ovarian cells but no follicles were added, as the latter were not required for this study design. Besides, follicles are precious material and their isolation is quite time-consuming. Use of human tissue for this study was approved by the Institutional Review Board of the Université Catholique de Louvain (IRB, 2012, 125). Informed consent was obtained before taking ovarian biopsies from 2 premenopausal women with regular cycles (48 and 49 years of age) undergoing laparoscopic surgery for benign gynecological disease. The tissue was initially processed as previously described [30, 31]. Cortical strips, sectioned into pieces of 0.5×0.5×1 mm using a McIlwain Tissue Chopper (Mickle Laboratory, UK), were incubated with 0.28Wünsch units/mL Liberase DH (Roche Diagnostics) for 75 min at 37 °C with gentle agitation. Tissue digestion was halted by addition of 10 ml of cold phosphate-buffered saline (PBS) supplemented with 10 % fetal bovine serum (FBS). The ovarian cell suspension was then successively filtered through sterilized 80 and 11 μm nylon net filters (Millipore, Brussels, Belgium). The obtained suspension was centrifuged (1200 rpm, 5 min) and the pellet resuspended in 500 μL of PBS supplemented with 10 % FBS. Ovarian

cells were counted in a Bürker chamber and their concentration adjusted to 10,000 cells/μL by centrifugation.

Leukemic cells

The BV-173 cell line was derived from a patient with Philadelphia chromosome-positive ALL (BCR-ABL b2-a2 fusion gene). This cell line was obtained from DSMZ (DSMZ no. ACC 20, Germany) and cultured in RPMI 1640 (+10 % FBS supplemented with antibiotic solution containing penicillin and streptomycin (Gibco). Repeated viability tests using a Bürker chamber to count cultured BV-173 cells in the course of successive passages always showed very high (~99 %) viability. A few microliters of the BV-173 cell suspension were added to a drop of fresh medium in a plastic petri dish. Using a stereomicroscope, 10 and 100 leukemic cells were retrieved with a 130 μm micropipette (Flexipet, COOK) and placed in separate medium droplets.

Incorporation of cells into a fibrin matrix

Formation of fibrin clots was done as previously described by Luyckx et al. [27]. A 12.5 μL fibrinogen droplet (Baxter, Belgium) was placed on a glass petri dish. Fifty thousand ovarian cells contained in 5 μL of medium and 10 or 100 leukemic cells (manually picked up with a micropipette under the stereomicroscope) were successively added to the fibrinogen. The fibrinogen droplet containing the cells was mixed with 12.5 μL of thrombin on a plastic petri dish and incubated for 45 min at 37 °C for fibrin polymerization. The clot was then gently detached for grafting.

For positive controls, 3 million BV-173 cells in 10 μL of medium were incorporated into the fibrin clots in a similar manner. A fibrin clot filled with cells is illustrated in Fig. 1a.

Xenografting of fibrin clots to SCID mice

Animal welfare guidelines were approved by the Committee on Animal Research of the Université Catholique de Louvain. Fourteen 6- to 9-week-old female SCID mice (Charles River Laboratories) were used for this study. Housing conditions, anesthesia and analgesia were previously described [26]. After performing a ventral midline incision, a peritoneal pocket was created on the right inner side of the peritoneum using non-absorbable 6–0 Prolene suture (Fig. 1b). The inner peritoneal surface was scratched with a scalpel [28], before gently pushing the fibrin clot into the peritoneal pocket and closing it with a stitch (Fig. 1c). Each mouse was grafted with a fibrin clot containing either 10 (5 mice), 100 (5 mice) or 3 million (4 mice) leukemic cells. Atipamezole (1 mg/kg; Antisedan, Pfizer) was used to reverse anesthesia after surgery.

Upon detection of a peritoneal mass or maximum 20 weeks after grafting, the mice were anesthetized using the same protocol as for the grafting procedure. Blood was retrieved by intracardiac puncture. They were then euthanized by cervical dislocation and bone marrow was collected by flushing femurs with MEM-HEPES containing 10 % FBS. The peritoneal grafts as well as the liver and spleen of each mouse were recovered. The samples were cut into 3 and assigned to PCR (Trizol Reagent), flow cytometry (MEM-HEPES + 10 % FBS) and microscopic analysis (formol).

Detection of leukemia in mice

Histological analysis

Tissue fragments (peritoneal grafts, liver and spleen) assigned to microscopic analysis were fixed in 4 % formaldehyde, embedded in paraffin, and serially sectioned (5 μm). Every third slide was stained with hematoxylin and eosin (Merck) for histological evaluation. The remaining slides (SuperfrostPlus, Menzel-Glaser) were kept for immunostaining.

Immunohistochemistry

Human anti-CD79α immunohistochemistry (IHC) was performed on recovered grafts and livers (12 sections/sample). CD79 is a transmembrane heterodimer (CD79α/CD79β) non-covalently associated with surface immunoglobulin forming the B-cell receptor complex required for antigen recognition.

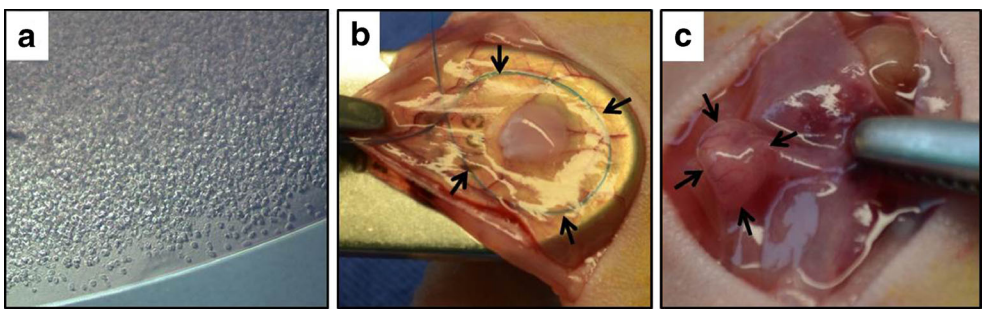

Fig. 1 (**a**) Leukemic cells embedded in a fibrin clot observed under the inverted microscope. (**b** and **c**) Grafting procedure and peritoneal bursa formation. *Black arrows* (**b**) show the suture used for preparation of the bursa, with the fibrin clot placed in the middle. The ends of the suture were pulled and closed with a knot to obtain a closed bursa with the fibrin clot inside (**c**)

CD79α is considered a pan-B cell marker and is expressed by BV-173 cells.

Sections were deparaffinized (Histosafe, Yvsolab SA) and rehydrated in 2-propanol (Merck). After blocking endogenous peroxidase activity with H2O2 0.3 %, a demasking step was performed for 75 min at 98 °C with citrate buffer and Triton X100. The sections were incubated for 30 min with 10 % normal goat serum (NGS) and 1 % bovine serum albumin (BSA, Sigma) to block non-specific binding sites, and then with mouse anti-human CD79α antibody, 1:30 dilution (Dako), at room temperature for 1 h. The slides were subsequently incubated with goat anti-mouse IgG (Envision, DAKO) for 60 min at room temperature. Diaminobenzidine was used as a chromogen (SK 4100, Vector Laboratories). Counterstaining was performed with hematoxylin before mounting the slides using DPX neutral mounting medium (Prosan, Merelbeke, Belgium). Negative controls consisted of the dilution solution without any anti-CD79α antibody. BV-173 cells were used as positive controls.

Flow cytometry analysis

All flow cytometry analyses were carried out in an accredited clinical flow cytometry laboratory specialized in onco-hematology.

Upon reception of the cell line and just before grafting, the complete BV-173 cell phenotype was verified by flow cytometry using a Navios (3-laser) flow cytometer, as routinely performed for acute lymphoid leukemia (ALL). For this purpose, we used 2 orientation tubes and 3 tubes for intra-cytoplasmic markers followed by 4 panels of 7 to 8 monoclonal antibodies for B-type ALL characterization. Then, based on the determined BV-173 cell phenotype, we selected a combination of markers (two 5-color panels, Fig. 3a) to allow differentiation of our cell line from other cells (notably mature lymphocytes) possibly present in our samples. Multiple markers were applied for more sensitive and specific cell detection. Conjugated reagents used for determining antigen expression were FITC-CD13 (SJ1D1), FITC-CD44 (J-173), PE-CD10 (J5-RD1), ECD-CD45 (J33), PC5-CD33 (D3HL60.251),PC5-CD38 (LS-198-4-3)and PC7-CD19 (J4.119), all from Immunotech, Beckman Coulter (BC). Both 5-color panels were utilized for each sample, the second panel to confirm the results of the first.

Each five-color panel was added to 100 μL of whole blood or 50 μL liver, spleen, peritoneal graft or bone marrow cell suspension after adjusting concentrations to $5–10\times10^3$ cells/μL. The cell and monoclonal antibody mixture was then gently vortexed and incubated for 30 min. Four milliliters of erythrocyte-lysing solution (for blood and bone marrow) or Isoton (for liver, spleen and peritoneal grafts) was added to each sample. The mixture was shaken and incubated for 10 min at room temperature. Finally, all the samples were centrifuged (300*g*, 5 min) at room temperature and the pellets resuspended in 1 mL of PBS.

For each sample, events were recorded over 300 s using a FC500 (BC) flow cytometer equipped with 2 lasers: a 488 argon laser and a 633 red HeNe laser. Flow-Check ™ fluorospheres (BC) and Flow-Check 675 (APC (675/633) Set-up Kit, BC) were used daily to assess flow cytometer optical alignment and fluidic system performance, while light scatter intensity and fluorescence intensity were controlled using Flow-Set ™ fluorospheres (BC) and Flow-Set 675 (APC (675/633) Setup Kit, BC), in order to evaluate optimal conditions for quantitative analysis of human leukocytes.

The CXP 2.2 software (BC) was used for both, acquisition and analysis purposes (listmode files). Cell targeting was carried out after manual gating around the mononucleated population on a forward vs. side-scatter dot plot. A second gate was then placed on the B lymphocyte (CD-19 positive) subpopulation (Fig. 3b–g), and the percentage of events expressing the BV-173 cell phenotype was analyzed. A minimum of 20 events was the cut-off for positivity.

PCR analysis: detection of the BCR-ABL fusion gene

For PCR analysis of mouse blood and bone marrow, erythrocytes were lysed. Leukocytes obtained by centrifugation were counted (Z1 Particle Counter, Coulter Corporation) and total RNA was extracted from 10^7 leukocytes with TriPure isolation reagent (Roche Diagnostics) according to the manufacturer's recommendations. Solid tissues were ground and placed in TRIzol reagent (Invitrogen) for RNA extraction. RNA concentrations were measured by fluorometry, and cDNA synthesis followed by quantitative RT-PCR were performed using the BCR-ABL Mbcr IS-MMR DX kit (Ipsogen) following the manufacturer's instructions. Briefly, cDNA synthesis was done with 1.5 μg of RNA in a volume of 15 μL, to which 22.5 μl of RT premix was added. After a first step at 25 °C for 10 min, then another at 37 °C for 60 min, reverse transcription was halted by inactivation at 85 °C for 10 min. Quantitative RT-PCR for each sample was performed in a final volume of 25 μL, containing 5 μL cDNA (200 ng of RNA equivalent). Thermal cycling was started with an initial denaturation step at 95 °C for 10 s, followed by 50 cycles at 95 °C for 5 s, then 60 °C for 30 s. Our samples and positive and negative controls were amplified in simplicate. Positive controls were commercial plasmid DNA calibrators containing target gene sequences. For negative controls, we used tissue from a healthy SCID mouse. Relative quantification was performed by quantification cycle (Cq) analysis and results were expressed as the ratio of the target gene (BCR-ABL fusion transcript gene) vs. the housekeeping gene (Abelson: ABL).

Results

Macroscopic analysis

All 4 mice grafted with 3×10^6 leukemic cells developed palpable peritoneal masses in the grafting site within 4 weeks. At this stage, however, the mice still looked healthy and did not show any clinical sign of terminal cancer (significant weight loss or cachexia, ruffled coat, hunching). The size of the masses is indicated in Table 1. Macroscopically, the masses were hard, white and well vascularized (Fig. 2a). Mice grafted with 10 or 100 leukemic cells did not develop any visible masses and were euthanized after 20 weeks. Upon macroscopic evaluation, the grafts were found to have shrunk in size as the fibrin was entirely degraded after 5 months. Because of their very little size, they were difficult to distinguish from the peritoneum and were identified by the stitch used to make the peritoneal bursa (as shown in Fig. 2d). None of the mice showed enlarged abdominal nodes or hepatosplenomegaly during dissection.

Histology and immunohistochemistry

Peritoneal masses Masses found in the 4 mice grafted with 3 million leukemic cells showed widespread invasion by lymphoblasts, all positive for anti-human CD79α (Fig. 2b).

Peritoneal grafts Grafted fibrin clots were identified at histology, then anti-human vimentin IHC was performed to identify human ovarian cells. Clots were present in fragments assigned to microscopic analysis in 6 of the 10 mice (3 grafted with 10 BV-173 cells and 3 grafted with 100 BV-173 cells), while in the remaining 4, it is likely that they were exclusively in fragments assigned to PCR and/or flow cytometry, as all recovered grafts were macroscopically cut into 3 pieces. In each case, the part of the clot subjected to microscopic analysis measured between 560 and 840 μm. They formed well vascularized structures somewhat resembling normal ovarian medullary tissue (Supplementary Fig 1). No signs of leukemic invasion were detected at histology and anti-human CD79α IHC performed on serial sections was also negative in all cases, suggesting an absence of human leukemia B cells in any of the grafts (Fig. 2e).

Mouse livers Three of the 4 mice grafted with 3 million leukemic cells showed liver invasion at classical light microscopy, and all 4 were positive for anti-human CD79α at IHC (Fig. 2c). Livers from mice grafted with 10 or 100 leukemic cells were all found to be disease-free at both histology and IHC (Fig. 2f).

Flow cytometry

BV-173 cell phenotype established by flow cytometry before grafting was as follows: CD19+,CD3-,CD20-, CD33+,

Table 1 Summary of results for leukemic cell detection by immunohistochemistry (IHC), flow cytometry (FC) and PCR in mice grafted with 10, 100 or 3 million leukemic cells

Grafted BV-173 cells	Mouse	Macroscopic peritoneal mass	Peritoneal graft			Liver			Spleen		Blood		Bone marrow	
			IHC	FC	PCR	IHC	FC	PCR	FC	PCR	FC	PCR	FC	PCR
10	1	no	−	−	−	−	−	−	−	−	−	−	−	−
	2	no	−	−	−	−	−	−	−	−	−	−	−	−
	3	no	−	−	−	−	−	−	−	−	−	−	−	−
	4	no	−	−	−	−	−	−	−	−	−	−	−	−
	5	no	−	−	−	−	−	−	−	−	−	−	−	−
100	6	no	−	−	−	−	−	−	−	−	−	−	−	−
	7	no	−	−	−	−	−	−	−	−	−	−	−	−
	8	no	−	−	−	−	−	−	−	−	−	−	−	−
	9	no	−	−	−	−	−	−	−	−	−	−	−	−
	10	no	−	−	−	−	−	−	−	−	−	−	−	−
3×106	11	yes (1.2×0.9×0.7 cm)	+	+	+	+	+	+	+	+	NP	+	NP	+
	12	yes (1×1×0.6 cm)	+	+	+	−	−	+	−	+	−	+	+	+
	13	yes (1×1×0.7 cm)	+	+	+	+	+	+	+	+	+	+	+	+
	14	yes (1×0.8×0.5 cm)	+	+	+	+	−	+	−	+	+	+	−	+
Neg ctrl	15	no	NA	NA	NA	−	NA	−	NA	−	NA	−	NA	−

Mice grafted with 10 or 100 leukemic cells showed no sign of leukemia in the different tissue samples by any of the techniques. All those grafted with 3 million leukemic cells developed leukemic masses in the grafting site and showed the presence of systemic disease.

NA not applicable, *NP* analysis not possible due to small sample volume

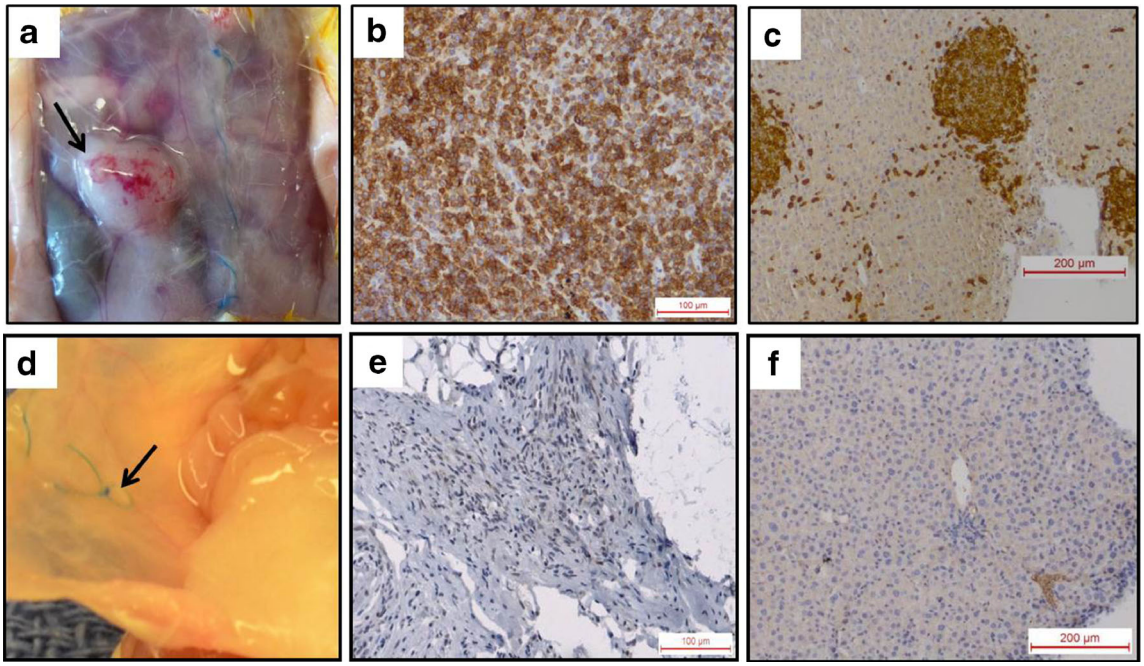

Fig. 2 **(a–c)** Macroscopic and IHC analysis of a mouse grafted with 3×10^6 leukemic cells. Peritoneal masses were palpable 4 weeks after grafting. The macroscopic view shows a hard, white and well vascularized peritoneal mass in the grafting site (**a**). Microscopic evaluation revealed widespread invasion of the mass by lymphocytic cells positive for anti-human CD79α (**b**). Microscopic analysis of the liver showed obvious leukemic invasion at histology, positive for anti-human CD79α immunostaining (**c**). **(d-f)** Macroscopic and IHC analysis of peritoneal grafts and liver recovered from a mouse implanted with 100 leukemic cells. The grafts had shrunk in size and were identified by the still present stitch in the peritoneal bursa (*black arrow*, **d**). No human CD79α-positive cells were identified in the grafts (**e**). The liver showed no sign of leukemic invasion at histology and was negative for anti-CD79α at IHC (**f**)

CD79α+, CD13+, CD10+, CD22+, CD43+, CD44+, CD45-, CD38+.

For the combination of markers selected for our experiments (two 5-color panels, Fig. 3a), among the CD19 positive mononucleated cell population, the following phenotypes corresponded to our BV-173 cells: panel 1: CD45-/CD13+/CD10+/CD33+; and panel 2: CD45-/CD10+/CD44+/CD38. The percentage of BV-173 cells found in each sample is detailed in Supplementary Table 1. In mice grafted with 10 or 100 leukemic cells, none of the tested samples showed any contamination by leukemic cells (Fig. 3e–g). Flow cytometry was not performed on blood or bone aspirate from mouse 11, as there was not enough tissue to conduct both tests. Since our previous results demonstrated that PCR was more sensitive than flow cytometry for this application, the entire samples were assigned to PCR. In the positive control group (3 million cells), however, peritoneal masses exhibited between 15 and 57 % BV-173 cells and at least one other tissue was positive in each mouse, proving that the disease was already systemic 4 weeks after grafting (Supplementary Table 1).

PCR analysis

BV-173 cells express BCR-ABL fusion transcripts, allowing detection of our cells with a sensitivity of 10^{-3} to 10^{-4}, depending on housekeeping gene expression (between 3200 and 32,000 ABL copies in our samples).

For the 4 mice grafted with 3×10^6 leukemic cells, all samples (peritoneal masses and all recovered tissues) were positive for the BCR-ABL leukemic marker (Table 1). Conversely, none of the samples from mice grafted with 10 or 100 leukemic cells showed any signs of leukemic cell contamination (Table 1).

Discussion

For patients with leukemia, malignant cells present in the bloodstream could also be present in the ovary at the time of cryopreservation [18]. There is currently no clinical alternative for these patients, whose only choice is OTC, but in whom transplantation cannot be performed due to the risk of reintroducing the disease. Fertility restoration techniques using follicles from cryopreserved tissue as a source of gametes are currently being investigated. In vitro follicle growth and oocyte maturation followed by in vitro fertilization could be an option. However, despite advances in the field, viable embryos and live offspring generated by this technique have only been achieved in mice so far [32–34], and major challenges remain to improve culture systems in humans to obtain competent human oocytes [35].

a

	FITC conjugates	*PE conjugates*	*ECD conjugates*	*PC5 conjugates*	*PC7 conjugates*
PANEL 1	CD13 -FITC	CD10 -PE	CD45 -ECD	CD33 –PC5	CD19 –PC7
PANEL 2	CD44 -FITC	CD10 -PE	CD45 -ECD	CD38 –PC5	CD19 –PC7

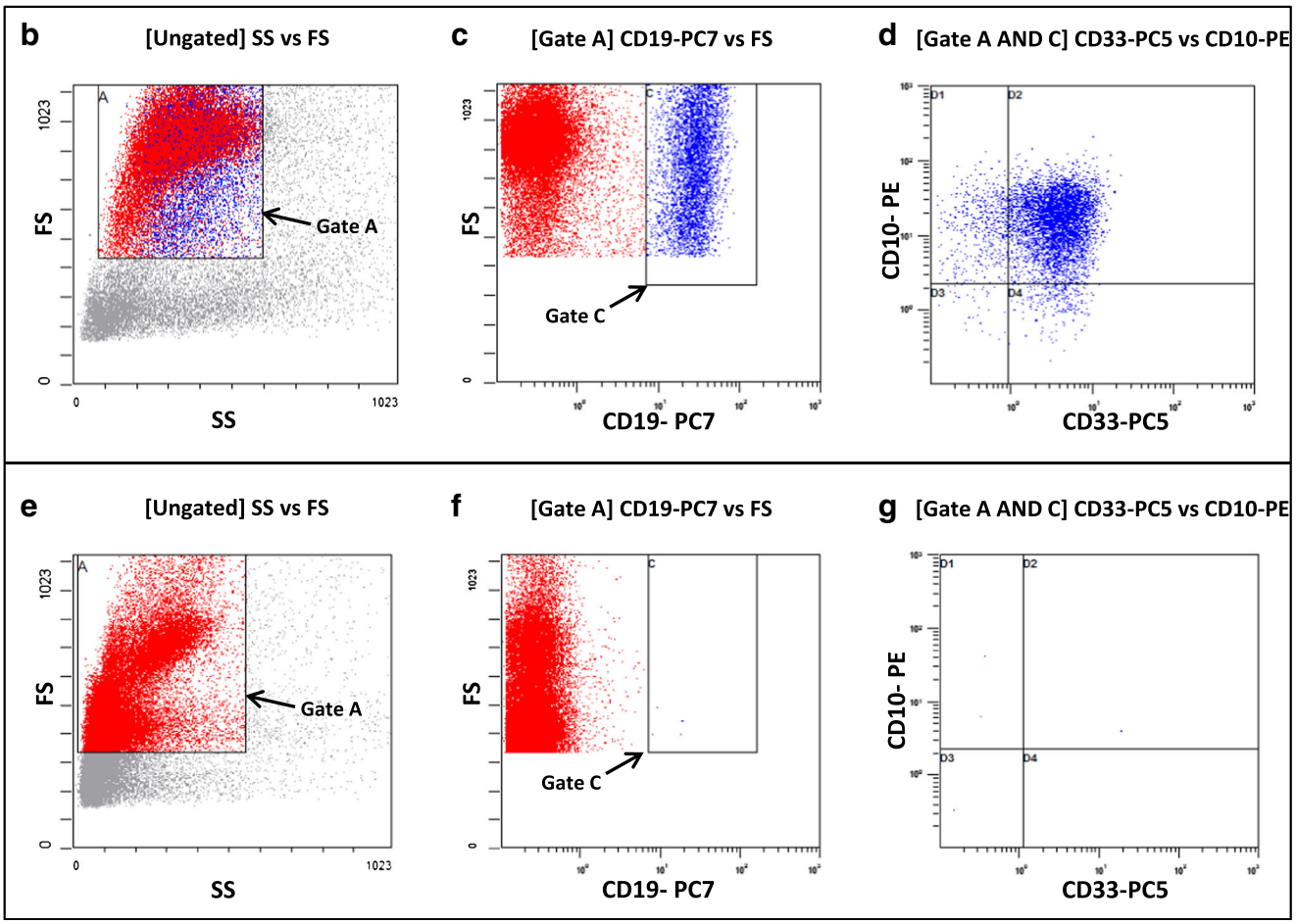

Fig. 3 (**a**) Two 5-color monoclonal antibody panels used for detection of the BV-173 cell line in our samples. (**b**–**g**) Gating strategy for flow cytometry detection of leukemic cells in bone marrow samples from a mouse grafted with 3×10^6 leukemic cells (**b**–**d**) and 100 leukemic cells (**e**–**g**). For the sake of clarity, only one example with panel 1 is shown in the figure. (**b** and **e**) Manual selection of the mononucleated population (gate A) on a bi-parametric dot plot with side scatter vs. forward scatter (**c** and **f**). Selection of CD19(PC7)-expressing cells (gate C) by a second manual gating. (**d** and **g**) Expression of CD33 and CD10 on a bi-parametric dot plot gated on A and C. BV-173 cells (CD19+, CD33+, CD10+) are colored in *blue* (**d**). In mice grafted with 10 or 100 leukemic cells, no BV-173 cells were identified (**g**)

The need to find alternative fertility-restoring solutions for these patients led us to investigate isolated follicle grafting and launch the artificial ovary concept. Follicles isolated from frozen-thawed ovarian tissue are embedded in 3-D matrices, such as plasma clots [24, 25], alginate [26] or fibrin [27, 28], along with ovarian stromal and endothelial cells, which allow formation of a well vascularized ovary-like structure [29]. This artificial ovary provides a more natural environment for follicle survival and development. Our group demonstrated, for the first time, the ability of isolated human preantral follicles embedded in a plasma clot to grow to the antral stage after xenotransplantation to mice [25]. Recent results on grafting of mouse follicles and ovarian cells in fibrin are also promising [28].

However, since isolated follicles are retrieved from potentially contaminated ovarian tissue suspensions, it is vital to ensure that suspensions are free of malignant cells before they are incorporated into the artificial ovary. Experiments were recently conducted in an in vitro model of ovarian tissue artificially contaminated with leukemic BV-173 cells, and a study on the safe isolation of follicles from tissue of leukemia patients is ongoing. The present study was initiated to investigate the leukemia-inducing potential of a few leukemic cells in an artificial ovary environment.

Several studies carried out in male rodent models have attempted to determine the number of leukemic cells in testicular tissue or testicular cell suspensions needed to

induce leukemia. Results differ between studies, but intratesticular [36] or intraperitoneal [37] injection of as few as 10 AML cells was found to induce disease in mice. The type of cell line used and its species-specific origin could also have an impact on the results. In a study using rat T leukemia cells [38], intratesticular injection of at least 20 cells was capable of inducing leukemia in rats within 21 days. When these same cells were injected intraperitoneally into nude mice, a minimum of 200 cells was required to induce leukemia [39]. These results indicate that host species and grafting site may influence the number of grafted cells and time required to induce leukemia. The microenviroment is also known to play an important role in tumor development [40], and interleukin-1, which is notably produced in the testis, may promote proliferation of leukemic cells in male.

No such study has yet been performed with ovarian tissue, and although cryopreserved ovarian tissue from leukemia patients was shown to induce tumors when grafted to SCID mice [18], the number of leukemic cells present in the tissue was not known. Our study is the first to evaluate the survival and tumor-inducing capacity of leukemic cells in an artificial ovary environment. The number of grafted leukemic cells grafted was based on results from a previous study using a model of artificially contaminated ovarian tissue, in which between 1 and 97 leukemic cells were found in the follicle suspension (Soares et al., submitted). We embedded these leukemic cells in a fibrin matrix along with 50,000 ovarian cells, as in our previous studies on the artificial ovary [26, 28]. In a clinical setting, the artificial ovary would be grafted, like cryopreserved ovarian cortex, either to the remaining ovary and/or inside a peritoneal window, both sites having proved equally effective [41, 42]. In our experiment, in order to mimic conditions inside the peritoneum, clots were grafted to its inner surface, inside a peritoneal pocket. Fibrin is known to provide a good environment for cell migration and proliferation, and serves as a reservoir of growth factors. Furthermore, it plays an important role in angiogenesis. Fingert et al. [43] reported that leukemic cells were able to survive and grow in a fibrin matrix when transplanted to the kidney capsule.

In our study, all mice grafted with 3×10^6 cells developed systemic disease within 4 weeks, but there was no sign of leukemia in mice grafted with 10 or 100 BV-173 cells after 20 weeks. In general, the rate of disease appearance is directly related to the number of transferred lymphoblasts [37, 44]. BV-173 cells have a doubling time of 30–48 h, so 5 months appeared more than adequate for our purposes. The maximum evaluation period in previous studies was 4 months [36].

Flow cytometry is a very sensitive method for leukemic cell detection. In our study, however, PCR proved more sensitive than flow cytometry, as certain samples negative at flow cytometry were found to be positive by PCR (mice 12 and 14). The lower sensitivity of our flow cytometry analysis was probably due to small sample volumes available for this technique, and also low levels of infiltration of certain samples by BV-173 cells. The scant cellularity of BV-173 cells could not therefore be compensated for by analysis of bigger volumes.

Because grafting of an artificial ovary is an avascular procedure, post-grafting ischemia, occurring before the revascularization process is activated, could explain the loss of a certain percentage of cells by hypoxia, be it ovarian or leukemic.

Moreover, the immunodeficient SCID mouse model used in this study is characterized by some residual immunity (natural killer cells, macrophages and granulocytes), which might confer a protective effect against tumor engraftment. Women in cancer remission are immunocompetent hosts, so their immune response may well act as an effective extrinsic tumor-suppressing system [45].

In a clinical setting, no risks should be taken and grafted follicles should be completely free of leukemic cells. We are currently working on the safe isolation of follicles from patients at risk of minimal residual disease.

Conclusion

This is the first study to evaluate the tumor-inducing potential of a few leukemic cells grafted inside an artificial ovary. Mice grafted with 10 or 100 BV-173 leukemic cells did not develop any sign of leukemia after 20 weeks. These results are reassuring as they suggest that as many as 100 BV-173 cells inadvertently grafted inside an artificial ovary are not capable of inducing leukemia, even in an immunodeficient xenografting model. However, in a clinical context, even the slightest risk should be avoided. Purging follicle suspensions of malignant cells is therefore a must to ensure a malignant cell-free follicle suspension before grafting.

Acknowledgments The authors thank Mira Hryniuk for reviewing the English language of the manuscript and Olivier Van Kerk and Dolores Gonzalez for their technical assistance. Help from specialized technicians, namely Patricia Leveugle and Anne-Marie Mazzon for flow cytometry experiments and Béatrice Delepaut for PCR, was also greatly appreciated.

This work was supported by grants from the Fonds National de la Recherche Scientifique de Belgique (5/4/150/5 and 7.4518.12F), Fonds Spéciaux de Recherche, Fondation Saint Luc, Foundation Against Cancer, and donations from Mr. Pietro Ferrero, Baron Frère, and Viscount Philippe de Spoelberch.

References

1. Edwards BK, Noone A-M, Mariotto AB, Simard EP, Boscoe FP, Henley SJ, et al. Annual Report to the Nation on the status of cancer, 1975–2010, featuring prevalence of comorbidity and impact on survival among persons with lung, colorectal, breast, or prostate cancer. Cancer. 2013:n/a-n/a.

2. Ward E, Desantis C, Robbins A, Kohler B, Jemal A. Childhood and adolescent cancer statistics, 2014. CA Cancer J Clin. 2014;64(2): 83–103.
3. Meirow D, Nugent D. The effects of radiotherapy and chemotherapy on female reproduction. Hum Reprod Update. 2001;7(6):535–43.
4. Wallace WH, Anderson RA, Irvine DS. Fertility preservation for young patients with cancer: who is at risk and what can be offered? Lancet Oncol. 2005;6(4):209–18.
5. Donnez J, Martinez-Madrid B, Jadoul P, Van Langendonckt A, Demylle D, Dolmans MM. Ovarian tissue cryopreservation and transplantation: a review. Hum Reprod Update. 2006;12(5):519–35.
6. Byrne J, Fears TR, Gail MH, Pee D, Connelly RR, Austin DF, et al. Early menopause in long-term survivors of cancer during adolescence. Am J Obstet Gynecol. 1992;166(3):788–93.
7. Larsen EC, Muller J, Schmiegelow K, Rechnitzer C, Andersen AN. Reduced ovarian function in long-term survivors of radiation- and chemotherapy-treated childhood cancer. J Clin Endocrinol Metab. 2003;88(11):5307–14.
8. Green DM, Kawashima T, Stovall M, Leisenring W, Sklar CA, Mertens AC, et al. Fertility of female survivors of childhood cancer: a report from the childhood cancer survivor study. J Clin Oncol. 2009;27(16):2677–85.
9. Anderson RA, Wallace WH. Antimullerian hormone, the assessment of the ovarian reserve, and the reproductive outcome of the young patient with cancer. Fertil Steril. 2013;99(6):1469–75.
10. Jadoul P, Dolmans MM, Donnez J. Fertility preservation in girls during childhood: is it feasible, efficient and safe and to whom should it be proposed? Hum Reprod Update. 2010;16(6):617–30.
11. Cancer in Children and Adolescents. Belgian Cancer Registry; 2013.
12. Dolmans MM, Jadoul P, Gilliaux S, Amorim CA, Luyckx V, Squifflet J, et al. A review of 15 years of ovarian tissue bank activities. J Assist Reprod Genet. 2013;30(3):305–14.
13. Dolmans MM, Luyckx V, Donnez J, Andersen CY, Greve T. Risk of transferring malignant cells with transplanted frozen-thawed ovarian tissue. Fertil Steril. 2013;99(6):1514–22.
14. Donnez J, Dolmans MM. Fertility preservation in women. Nat Rev Endocrinol. 2013;9(12):735–49.
15. Cobo A, Garcia-Velasco JA, Domingo J, Remohi J, Pellicer A. Is vitrification of oocytes useful for fertility preservation for age-related fertility decline and in cancer patients? Fertil Steril. 2013;99(6): 1485–95.
16. Cakmak H, Rosen MP. Ovarian stimulation in cancer patients. Fertil Steril. 2013;99(6):1476–84.
17. Meirow D, Hardan I, Dor J, Fridman E, Elizur S, Ra'anani H, et al. Searching for evidence of disease and malignant cell contamination in ovarian tissue stored from hematologic cancer patients. Hum Reprod. 2008;23(5):1007–13.
18. Dolmans MM, Marinescu C, Saussoy P, Van Langendonckt A, Amorim C, Donnez J. Reimplantation of cryopreserved ovarian tissue from patients with acute lymphoblastic leukemia is potentially unsafe. Blood. 2010;116(16):2908–14.
19. Rosendahl M, Andersen MT, Ralfkiaer E, Kjeldsen L, Andersen MK, Andersen CY. Evidence of residual disease in cryopreserved ovarian cortex from female patients with leukemia. Fertil Steril. 2010;94(6): 2186–90.
20. Amiot C, Angelot-Delettre F, Zver T, Alvergnas-Vieille M, Saas P, Garnache-Ottou F, et al. Minimal residual disease detection of leukemic cells in ovarian cortex by eight-color flow cytometry. Hum Reprod. 2013;28(8):2157–67.
21. Greve T, Clasen-Linde E, Andersen MT, Andersen MK, Sorensen SD, Rosendahl M, et al. Cryopreserved ovarian cortex from patients with leukemia in complete remission contains no apparent viable malignant cells. Blood. 2012;120(22):4311–6.
22. Bastings L, Beerendonk CC, Westphal JR, Massuger LF, Kaal SE, van Leeuwen FE, et al. Autotransplantation of cryopreserved ovarian tissue in cancer survivors and the risk of reintroducing malignancy: a systematic review. Hum Reprod Update. 2013;19(5):483–506.
23. Rosendahl M, Greve T, Andersen CY. The safety of transplanting cryopreserved ovarian tissue in cancer patients: a review of the literature. J Assist Reprod Genet. 2013;30(1):11–24.
24. Dolmans MM, Martinez-Madrid B, Gadisseux E, Guiot Y, Yuan WY, Torre A, et al. Short-term transplantation of isolated human ovarian follicles and cortical tissue into nude mice. Reproduction. 2007;134(2):253–62.
25. Dolmans MM, Yuan WY, Camboni A, Torre A, Van Langendonckt A, Martinez-Madrid B, et al. Development of antral follicles after xenografting of isolated small human preantral follicles. Reprod BioMed Online. 2008;16(5):705–11.
26. Vanacker J, Luyckx V, Dolmans MM, Des Rieux A, Jaeger J, Van Langendonckt A, et al. Transplantation of an alginate-matrigel matrix containing isolated ovarian cells: first step in developing a biodegradable scaffold to transplant isolated preantral follicles and ovarian cells. Biomaterials. 2012;33(26):6079–85.
27. Luyckx V, Dolmans MM, Vanacker J, Scalercio SR, Donnez J, Amorim CA. First step in developing a 3D biodegradable fibrin scaffold for an artificial ovary. J Ovarian Res. 2013;6(1):83.
28. Luyckx V, Dolmans MM, Vanacker J, Legat C, Fortuno Moya C, Donnez J, et al. A new step toward the artificial ovary: survival and proliferation of isolated murine follicles after autologous transplantation in a fibrin scaffold. Fertil Steril. 2014;101(4):1149–56.
29. Dath C, Dethy A, Van Langendonckt A, Van Eyck AS, Amorim CA, Luyckx V, et al. Endothelial cells are essential for ovarian stromal tissue restructuring after xenotransplantation of isolated ovarian stromal cells. Hum Reprod. 2011;26(6):1431–9.
30. Vanacker J, Camboni A, Dath C, Van Langendonckt A, Dolmans MM, Donnez J, et al. Enzymatic isolation of human primordial and primary ovarian follicles with Liberase DH: protocol for application in a clinical setting. Fertil Steril. 2011;96(2):379–83.e3.
31. Dolmans MM, Michaux N, Camboni A, Martinez-Madrid B, Van Langendonckt A, Nottola SA, et al. Evaluation of Liberase, a purified enzyme blend, for the isolation of human primordial and primary ovarian follicles. Hum Reprod. 2006;21(2):413–20.
32. Eppig JJ, O'Brien MJ. Development in vitro of mouse oocytes from primordial follicles. Biol Reprod. 1996;54(1):197–207.
33. O'Brien MJ, Pendola JK, Eppig JJ. A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. Biol Reprod. 2003;68(5): 1682–6.
34. Spears N, Boland NI, Murray AA, Gosden RG. Mouse oocytes derived from in vitro grown primary ovarian follicles are fertile. Hum Reprod. 1994;9(3):527–32.
35. Telfer EE, Zelinski MB. Ovarian follicle culture: advances and challenges for human and nonhuman primates. Fertil Steril. 2013;99(6): 1523–33.
36. Hermann BP, Sukhwani M, Salati J, Sheng Y, Chu T, Orwig KE. Separating spermatogonia from cancer cells in contaminated prepubertal primate testis cell suspensions. Hum Reprod. 2011;26(12): 3222–31.
37. Fujita K, Ohta H, Tsujimura A, Takao T, Miyagawa Y, Takada S, et al. Transplantation of spermatogonial stem cells isolated from leukemic mice restores fertility without inducing leukemia. J Clin Invest. 2005;115(7):1855–61.
38. Jahnukainen K, Morris I, Roe S, Salmi TT, Makipernaa A, Pollanen P. A rodent model for testicular involvement in acute lymphoblastic leukaemia. Br J Cancer. 1993;67(5):885–92.
39. Hou M, Andersson M, Zheng C, Sundblad A, Soder O, Jahnukainen K. Decontamination of leukemic cells and enrichment of germ cells from testicular samples from rats with Roser's T-cell leukemia by flow cytometric sorting. Reproduction. 2007;134(6): 767–79.

40. Fujita K, Tsujimura A, Hirai T, Ohta H, Matsuoka Y, Miyagawa Y, et al. Effect of human leukemia cells in testicular tissues grafted into immunodeficient mice. Int J Urol. 2008;15(8):733–8.
41. Donnez J, Jadoul P, Pirard C, Hutchings G, Demylle D, Squifflet J, et al. Live birth after transplantation of frozen-thawed ovarian tissue after bilateral oophorectomy for benign disease. Fertil Steril. 2012;98(3):720–5.
42. Donnez J, Dolmans MM, Pellicer A, Diaz-Garcia C, Sanchez Serrano M, Schmidt KT, et al. Restoration of ovarian activity and pregnancy after transplantation of cryopreserved ovarian tissue: a review of 60 cases of reimplantation. Fertil Steril. 2013;99(6):1503–13.
43. Fingert HJ, Chen Z, Mizrahi N, Gajewski WH, Bamberg MP, Kradin RL. Rapid growth of human cancer cells in a mouse model with fibrin clot subrenal capsule assay. Cancer Res. 1987;47(14):3824–9.
44. Jahnukainen K, Hou M, Petersen C, Setchell B, Soder O. Intratesticular transplantation of testicular cells from leukemic rats causes transmission of leukemia. Cancer Res. 2001;61(2):706–10.
45. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature. 2001;410(6832):1107–11.